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Development: Facial Makeup Enhancing Our Looks

A recent study in mice deciphers the complex genetic regulatory network underlying the morphogenesis of the face. The enhancer landscape underlying craniofacial development provides multiple entry points to understand what makes up the face, in natural variation or pathological conditions.

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A common parenting tale is that newborn faces tend to resemble more the faces of their fathers than the faces of their mothers. Indeed, there might actually be an evolutionary reason behind this: fathers can never be completely sure that the offspring is really theirs and are thus more likely to invest in childcare when they recognize themselves in their children [1]. Although this is probably an urban legend and babies resemble both their parents equally [2,3], it betrays the fact how important faces are for recognizing one another and how acutely our brain is tuned to notice even slight variations in facial proportions. Recognizing and focusing on faces has become socially vital for us humans, so critical that we even see faces where there are none — like the face on Mars, the man in the Moon, or the Virgin Mary on a grilled cheese sandwich [4]. But despite an age-old interest in our looks, the genetic basis of what shapes our face is largely unknown. This is even more surprising giving that facial characters are among the most heritable traits in humans — just think how much identical twins resemble each other — and facial malformations are among the most frequently occurring birth defects. Now, a recent paper by Attanasio *et al.* [5] addresses this long-standing question in mice using a combination of molecular biology, genetic, and morphometric approaches (Figure 1). The authors focus their attention on distant-acting transcriptional enhancers, rather than coding changes in genes, reasoning that these elements would be more adept at generating the subtle changes observed between different individual faces. Moreover, many developmental regulators are known to have pleiotropic effects, and changes in transcriptional circuitries provide an elegant solution to diversify gene function in both space and time, while preserving their ancestral tasks [6].

Even though humans might not as readily discern subtle changes in murine facial features, the authors nevertheless chose the laboratory mouse to tackle these questions, given the multitude of molecular and genetic tools available in this model organism. Previous studies had successfully predicted enhancer sequences through the identification of genomic regions bound by the transcriptional co-regulator p300 [7,8]. Following this rationale, Attanasio *et al.* isolated embryonic tissue from the faces of mouse embryos during development and performed ChIP-seq analysis, to locate p300-bound genomic sequences that thus were suspected to be enhancers active during facial morphogenesis.

Overall, they identified over 4300 putative enhancers bound by p300. These candidate sequences were located up to 1.4 Mb (median distance 44 kb) away from the next known transcriptional start site, in line with the notion that such regulatory elements can act over very large genomic distances [9]. 87.5% of the identified sequences showed evolutionary constraint, with 96.7% being conserved in humans. But more importantly for this study, genes neighboring these enhancers were more likely to be already known to be involved in craniofacial development and birth defects than by chance alone.

Both criteria, sequence conservation and involvement in craniofacial development, were subsequently used to narrow down the list of candidate enhancers to 205 for more in-depth follow-up analyses. To verify their impact on transcriptional regulation, the authors performed transient transgenic reporter assays in mice. Each candidate enhancer was cloned upstream of a minimal promoter potentially driving the expression of a LacZ reporter gene *in vivo*, if the tested element has enhancer activity [10]. Even with stringent criteria, an astounding 59% of the over 200 tested transgenes showed expression specific to craniofacial areas. In addition to these elements, the authors added another 75 from previous studies and analyzed them in great detail using optical projection tomography to document the observed patterns in a 3D atlas of craniofacial enhancer activity (some of the beautiful resulting movies can be seen in the supplemental material and all of the data are available on https://www.facebase.org/).

Interestingly, despite Attanasio *et al.* [5] testing individual enhancers, the resulting expression patterns were quite complex. There was no simple formula whereby one enhancer equals one anatomical structure. Quite the contrary, some enhancers drove expression in multiple tissues, some enhancers just in substructures — such as in a part of the nose or a part of the jaw. Importantly, however, the expression of reporters driven by the putative enhancers overlapped in most cases with the expression of the most closely located gene, allowing for the functional dissection of enhancer landscapes of individual genes. In a beautiful example for the gene Msx1, the authors could show that seven independent enhancers drove individual patterns that partially recapitulated the endogenous expression of Msx1 itself. These results provide stunning support for the textbook notion of the fine tuning potential that enhancers can provide.

The data derived from the study of Attanasio *et al.* [5] are likely to have direct implications on human disease as well. Association studies have found many areas in the genome linked to craniofacial birth defects like cleft palate or cleft lip. In many cases, however, these areas are devoid of genes, which makes finding the actual mutation underlying the disease very hard. This list of over 4000 craniofacial enhancers will be a gold mine for human geneticists to add to the spectrum of candidate loci.

What sets the study of Attanasio *et al.* [5] apart from others, however, is the fact that the authors functionally tested some of the enhancers by removing them from the mouse genome and analyzing the resulting functional consequences. Previous work from the authors and others has

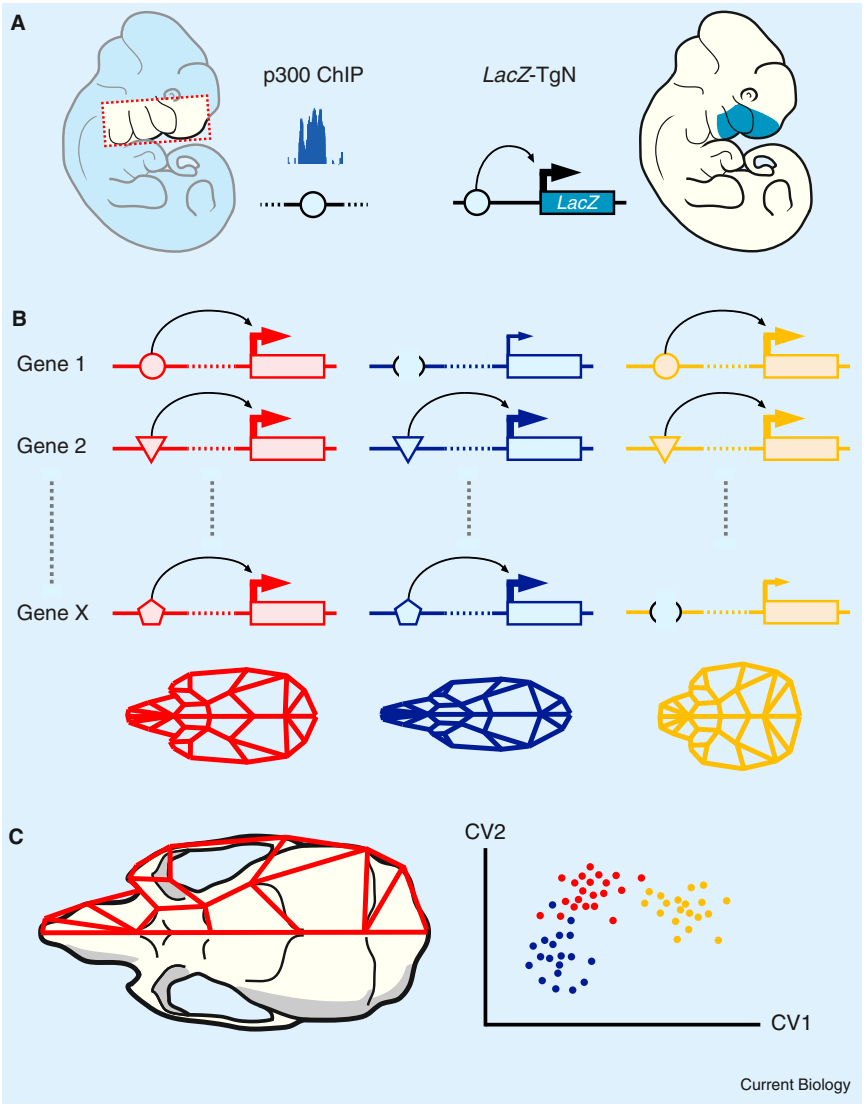

Figure 1. Characterizing the enhancer landscape underlying craniofacial diversity.

(A) Molecular identification of potential enhancers active during craniofacial morphogenesis using p300 ChIP-seq analysis of embryonic face tissue and LacZ transgene reporter assays. (B) Functional analysis of candidate enhancers. Regulatory elements (square, triangle, pentagon) are individually removed from the mouse genome by gene targeting strategies. Their impact on skull morphogenesis is evaluated measuring the resulting changes in gene expression (rectangle, arrow), as well as using morphometric analyses on defined landmarks of CT-scan images. (C) Although the overall phenotypes seem subtle at first glance, canonical variate (CV) analysis reveals significant changes in skull morphology, partly continuous with the observed wild-type spectrum of variation.

revealed a plethora of different enhancer elements from other tissues like the brain, the heart or the limb [11–14]. What these studies lacked, however, was the functional analysis of a knockout in the mouse. One reason previous studies avoided such analyses might have been previous negative results obtained with ultra-conserved elements that gave no obvious phenotype when mutated [15]. Terms like 'redundancy' and 'shadow enhancers' were invoked to account for the lack of obvious phenotypes. However, Attanasio *et al.* [5] did not shy away and found that the removal of the enhancers that emerged in their study not only resulted in a decrease of the expression of their suspected target genes, but also changed the skull shape of the mice. Although no clear phenotype was detectable at first in adult mouse skulls, the authors subsequently observed small, but significant phenotypic changes by measuring multiple standardized skeletal landmarks on micro computer-tomography generated images. Interestingly, the overall phenotypes observed were less severe than the pathological changes observed upon deletion of the gene itself. By going the distance with this in-depth analyses, the authors thereby not only provided another proof for the power of regulatory evolution to drive subtle morphological changes using pleiotropic genes [16], but also set the benchmark against which future enhancer analysis will have to be measured.

With the study of Attanasio *et al.* [5] a picture emerges of multiple enhancer elements driving the expression of target genes in overlapping and potentially redundant fashion, which most likely will hold true for a variety of other genes and developmental contexts. This dataset, on the one hand, thus provides us with a road-map to study the regulatory genome at a molecular and functional level, while at the same time might help to explain the phenotypic variation seen in human faces. We are indeed finely tuned to recognize such facial variation; with the new insight in hand we may finally be equipped to also understand how it comes about.

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Development: A Deep Breath for Endocrine Organ Evolution

Developmental biologists have made surprising discoveries on the evolutionary origins of cell types, organs and body plans. Now, an elegant study in *Drosophila* raises interesting questions about the origin of two major endocrine organs of insects.

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Although evolution is a continuous process, when we take a large-scale view certain evolutionary transitions stand out as milestones in the evolution of body form and function. We recognize events, such as the invention of muscles and neurons, or the evolution of segmented bodies, hormonal control systems and centralized brains as major changes, and use them to categorize the animals into discrete phyla with more or less distinctive 'body plans'. While we are certain that these transitions did take place in deep branches of the animal tree, we often do not understand how they occurred and have difficulty in reconstructing functional intermediate steps taken during these transitions. We know that vertebrates, insects, mollusks and sea anemones are all related to each other, but we cannot yet picture what their common ancestors looked like and how they lived. The deepest ancestral forms and functions of animals are the 'known unknowns' of animal evolution. New work by Sánchez-Higueras, Sotillos and Castelli-Gair Hombría [1], published in this issue of *Current Biology*, raises new questions about one of these unknowns — the evolution of arthropod endocrine systems — by revealing an unexpected link between two major endocrine glands and the respiratory organs (tracheae) of insects.

Arthropods — including insects, crustaceans, spiders, centipedes and the extinct trilobites — are among the most successful animals on earth: insects alone comprise more than half of all known living species (Haldane once said "the Creator, if he exists, has a special preference for beetles"); ants represent the largest part of animal biomass in rainforests, as do copepods and krill in the oceans. All arthropods are characterized by the possession of a hardened exoskeleton and a modular body consisting of repeated (but not necessarily identical) segments. The exoskeleton is likely to have been a key feature in the establishment of the arthropod body plan — it provides protection and leverage for muscles, but also constrains growth and the exchange of respiratory gases and ions with the environment. The arthropod ancestors evolved solutions to these constraints, which involved the endocrine and respiratory organs that are the focus of the Sánchez-Higueras *et al.* [1] paper.

To escape the constraints on growth, arthropods exploited moulting, which allows them to replace the exoskeleton by a larger one as the body grows. Two hormones became tightly associated with growth through moulting: ecdysone, whose levels in the blood (hemolymph) provide the signal for moulting, and juvenile hormone, whose action maintains the juvenile characteristics and prevents metamorphosis during successive larval moults [2–4]. In insects, two endocrine glands produce and release these hormones into the hemolymph: the prothoracic gland releases ecdysone and the corpus allatum releases juvenile hormone.

To escape the constraints imposed by the impermeable cuticle, many arthropods also evolved specialized surfaces for gas and ion exchange. In the aquatic ancestors of arthropods and in today's crustaceans, these functions are often carried out by specialized appendages called gills. In terrestrial arthropods, such as centipedes, spiders and insects, the respiratory function is carried out by internal respiratory organs, called book lungs and tracheae, respectively. There is evidence suggesting that the book lungs and tracheae of some terrestrial arthropods evolved from the gills of their aquatic ancestors, by internalization of these respiratory surfaces into the body [5–7].

Until now endocrine glands and tracheae were supposed to be unrelated; populations of cells that evolved independently in response to different adaptive pressures on the arthropod body plan. The results of Sánchez-Higueras *et al.* [1] question this view, by demonstrating that the embryonic primordia of the corpus allatum and the prothoracic gland are serially homologous to the primordia of tracheal cells. Serial homology means that these structures originate from identical groups of cells located in successive segments of the body, which are defined by a common set of developmental instructions; as is the case for successive limbs in insects or vertebrae in mammals. Serial